Protistology 16 (3): 236–245 (2022) | doi:10.21685/1680-0826-2022-16-3-8

Protistology

LETTERS TO THE EDITOR

Amoebozoan barcoding marker cytochrome c oxidase (Cox1), RNA editing and issues in creating a public reference sequence database

Alexander A. Kudryavtsev

Laboratory of Cellular and Molecular Protistology, Zoological Institute of the Russian Academy of Sciences, 199034 Saint Petersburg, Russia

| Submitted May 25, 2022 | Accepted August 6, 2022 |

Summary

DNA barcoding using a fragment of the mitochondrial cytochrome c oxidase subunit 1 (Cox1) gene is a promising tool not only in animals but also for many groups of protists, including Amoebozoa. To use this tool, we need a reference database for the comparison and assignment of newly obtained sequences. As NCBI/GeneBank® is the most complete molecular sequence database to date, it is logical to use it as a reference database. In fact, it is used as such, when the newly obtained sequences are checked against this database using BLAST. Yet, a quarter of all available barcoding Cox1 sequences of Amoebozoa would not be seen in the BLAST results, as they are deposited with the status 'UNVERIFIED'. Some of these sequences show reading frame shifts due to multiple single nucleotide deletions. These deletions, seen at the genomic level, may indicate presence of insertional RNA editing in this gene. This phenomenon was experimentally proven only in myxomycetes and Arcellinida among Amoebozoa. Interestingly, many sequences marked as UNVERIFIED do not show frame shifts or other signs of RNA editing, while some of the sequences that are not assigned this status, do. For the sequence database to be fully searchable, new sequences have to be properly accessioned. A recent communication with NCBI confirms that when a sequence has putative editing sites, the submitter should provide a note on this feature and references to appropriate papers. In this case, the sequence can be accessioned normally.

Key words: Amoebozoa, cytochrome c oxidase, DNA barcoding, NCBI/GenBank, reference sequence database

Cytochrome c oxidase subunit 1 (Cox1) gene as a DNA barcoding marker for Amoebozoa

The DNA barcoding was initially developed as the approach to species identification in living organisms based on DNA sequences (Hebert et al., 2003a, 2003b; Hebert and Gregory, 2005). As reviewed in DeSalle and Goldstein (2019), DNA barcoding has grown during the two decades into a powerful research area with almost 4000 papers published and applications in fundamental research from alpha-taxonomy to ecology and community structure analysis, and in practice, from conservation biology to forensics. Primarily, DNA barcoding was

https://doi.org/10.21685/1680-0826-2022-16-3-8

Corresponding author: Alexander A. Kudryavtsev. Laboratory of Cellular and Molecular Protistology, Zoological Institute of the Russian Academy of Sciences, Universitetskaya Emb., 1, 199034 Saint Petersburg, Russia; alexander.kudryavtsev@zin.ru

developed for traditional 'kingdoms' of multicellular organisms, i.e., metazoans, green plants, and fungi. Different molecular markers were proposed for these groups. The earliest of the DNA barcodes was ca. 700 b.p. long part of the mitochondrial cytochrome c oxidase subunit 1 gene (Cox1) proposed as a DNA barcode for animals (Hebert et al., 2003a). The choice of this particular locus is based on the fact that the Cox1 gene is among the most conservative protein-coding genes in animals (Brown, 1985). This allowed the construction of universal primers for PCR amplification of this fragment (Folmer et al., 1994). The data accumulated since this publication became a basis for the founding work of Hebert et al. (2003a) where it was shown that this fragment allowed high-level taxa identification based on the amino acid sequences, and species identification based on the nucleotide sequences. Later on, plastid loci rbcL and matK were recommended for land plants (CBOL Plant Working Group, 2009), and nuclear internal transcribed spacers (ITS) – for fungi (Seifert, 2009; Begerow et al., 2010; Schoch et al., 2012).

The molecular genetic diversity of protists is much broader than that of the above-mentioned kingdoms. Animals, fungi, and plants branch in derived positions in more inclusive Opisthokonta and Archaeplastida (Adl et al., 2019). In terms of genetic diversity, protists are almost equivalent to all eukaryotes. Therefore, it is clearly unrealistic to expect a single or even a few DNA barcoding markers for all protists. Therefore, CBOL Protist Working Group (ProWG) proposed a two-step approach to DNA barcoding of protists (Pawlowski et al., 2012). The main idea behind this approach is to use a V4 variable region of small-subunit (SSU) ribosomal RNA gene as a universal barcode for preliminary identification followed by application of group-specific barcodes designated depending on a specific clade where the species being identified belong. Several studies that specifically addressed group-specific barcodes for lobose amoeboid protists (Amoebozoa) mostly came to a consensus that a mitochondrial locus, a partial sequence of the cytochrome c oxidase subunit 1 (Cox1) gene, is the most perspective DNA barcode for this clade. After a pioneering study by Nassonova et al. (2010) on members of the genus *Vannella* Bovee, 1965 (Flabellinia), the utility of this marker was demonstrated in arcellinid testate amoebae (Kosakyan et al., 2012, 2013, 2015, 2016; Singer et al., 2015; Duckert et al., 2018, 2021; González-Miguéns et al., 2022), as well as in the genera *Cochliopodium* Hertwig and Lesser, 1874 (Himatismenida; Geisen et al., 2014; Tekle, 2014; Kudryavtsev et al., 2021), *Korotnevella* Goodkov, 1988, and *Neoparamoeba* Page, 1987 (Dactylopodida; Zlatogursky et al., 2016; Hansen et al., 2019; Udalov and Völcker, 2022). Some sequences without a detailed analysis of barcoding power were obtained also for naked Tubulinea (*Copromyxa* and *Saccamoeba*; Kostka et al., 2017), several more Dactylopodida (*Cunea* Kudryavtsev and Pawlowski, 2015, *Paramoeba* Schaudinn, 1896, *Pseudoparamoeba* Page, 1979, and *Vexillifera* Schaeffer, 1926; Kudryavtsev and Pawlowski, 2015; Kudryavtsev et al., 2018; English et al., 2019; Volkova et al., 2019; Kudryavtsev and Volkova, 2020; Kudryavtsev et al., 2020; Udalov et al., 2020), several more Vannellida (species of *Vannella, Clydonella* Sawyer, 1975, *Paravannella* Kudryavtsev, 2014, and *Ripella* Smirnov et al., 2007; Kudryavtsev, 2014; Kudryavtsev and Gladkikh, 2017; Kudryavtsev and Volkova, 2018; Kudryavtsev et al., 2019), and *Squamamoeba* (Cutosea; Kudryavtsev and Pawlowski, 2013). In addition to the taxa mentioned, quite a few sequences were deposited in NCBI/GenBank® for Acanthopodida (*Acanthamoeba* and *Balamuthia*) and slime molds (Dictyostelia and Myxogastria). In *Dictyostelium*, portions of mitochondrial DNA including cytochrome c oxidase were analyzed together with nuclear markers (Douglas et al., 2011) to evaluate species borders and distribution patterns, while in Myxogastria, the primary interest in this gene was due to the editing processes that occur in their mitochondria (e.g. Gott et al., 1993; Horton and Landweber, 2000; Traphagen et al., 2010). Furthermore, the analysis of the Cox1 gene was part of the DNA barcoding, alpha taxonomic and population studies in this group (Feng and Schnittler, 2015; Liu et al., 2015; Shchepin et al., 2017, 2022; Bortnikov et al., 2018). In Variosea, Cox1 gene sequences were obtained only as parts of mitochondrial genomes in *Phalansterium* (Pombert et al., 2013), *Protostelium* and *Planoprotostelium*, but otherwise no data are available. It is noteworthy that outside Variosea, a number of complete mitochondrial genomes of Amoebozoa are available in the database, namely for Vannellida, Dactylopodida, *Thecamoeba, Vermamoeba*, and *Acanthamoeba* (Fučíková and Lahr, 2016; Bondarenko et al., 2022; reviewed in Bondarenko et al., 2019).

A reference database of Cox1 and challenges for its creation

In the majority of studies where its barcoding properties were evaluated, Cox1 was recognized as a promising DNA marker for Amoebozoa. Therefore, a really needed action for the practical application of this marker as a barcode is the creation of a reference database, i.e., a curated set of annotated sequences from reliably identified and/or properly described morphospecies. Such dataset can be used to identify and assign new sequences, and analyze potential metabarcoding data. Ideally, this reference dataset should be a part of publicly available sequence databases such as NCBI/Genbank® (Sayers et al., 2022). This facilitates a broad comparison of new sequences with the existing ones using powerful search and comparison tools, for example, BLAST (Altschul et al., 1990). Two points are important to mention here. First, the NCBI/Genbank® is the broadest and most comprehensive molecular sequence database to date, used by anyone who obtains new sequence data. It is compulsory to deposit newly published molecular sequences in this database, which is commonly used as a reference database by anyone who obtains new sequences to check the identity of the newly obtained data. Second, BLAST is a universal and widely distributed search tool implemented in many packages and databases. Therefore, BLAST search of a new sequence against NCBI/Genbank® allows researchers to verify simultaneously whether the correct locus was sequenced, and how it is related to the other, already available sequences. However, detailed analysis of the contents of the NCBI/Genbank® database with respect to the Cox1 gene sequences of Amoebozoa shows that the available database that could serve as a reference set is still far from complete, while part of it is invisible when it comes to the BLAST search. The main issues of this database are analyzed and described below.

The DNA sequences of the Amoebozoa Cox1 gene, including those contained within the full mitochondrial genomes, were downloaded from NCBI and aligned using MAFFT (Katoh and Standley, 2013) with default parameters. The choice of an appropriate translation table, translation, and analysis of reading frame shifts were performed using Seaview v. 5.0.1 (Gouy et al., 2021). Alignment is available from the author on request. In total, the alignment of the amoebozoan Cox1 gene sequences downloaded from NCBI on August 3, 2022 contained 1578 sequences, including 18 sequences published as part of the complete mitochondrial genomes. Among them, 388 sequences had an UNVERIFIED status in the database (Table 1). This status in the NCBI/GenBank® database is assigned to the sequences that have no valid sequence features. According to 'The GenBank Submission Handbook' (https://www.ncbi.nlm.nih.gov/books/NBK566993/), 'Feature annotation is the addition of biological features such as genes and associated coding regions, structural RNA, variation information, exon, introns, etc. to your submitted sequence. The annotation should include the location of the feature (start and stop) and a description of the feature.' Even if the features are provided with the submission, GanBank staff members manually verify them and can argue that the features provided by the submitter are not verifiable. In this case, a comment to the sequence data appears with the statement "GenBank staff is unable to verify sequence and/or annotation provided by the submitter". Such sequences are marked as UNVERIFIED and, most importantly, become "invisible" for the BLAST search, as they are not included in the corresponding databases. An obvious drawback of this situation is the lack of possibility for researchers to fully check the identity of their newly obtained sequences.

Putative RNA editing, reading frame shifts, and Cox1 database

Detailed analysis of the published Cox1 sequences with UNVERIFIED status and the author's own experience show that most of them have problems with their reading frames when analyzed at the genomic DNA level. In particular, some of the sequences may undergo post-transcriptional modifications (i.e., RNA editing). In this case, a correct reading frame appears only in mRNA, whereas genomic sequence would demonstrate frame shifts, specifically if insertional editing is involved. Even without experimental evidence of this editing, its presence may be reasonably suggested when sequence quality is satisfactory, multiple sequences obtained from the same organism are identical, and the deletions are evident when sequences in question are compared to other sequences in an alignment. RNA editing is known for Amoebozoa and seems to be usual in some groups where it was proven experimentally. For example, multiple sites of insertional editing and base conversion were detected in *Physarum polycephalum* and

Table 1. Quantitative characteristics of the publicly available database of amoebozoan Cox1 gene sequences in NCBI/GenBank®.

Major clade	Genus (species)	Total Cox1 sequences	Unverified Cox1 sequences
Flabellinia	*Cunea*	5	–
	Korotnevella	55	51
	Neoparamoeba	45+1*	8
	Paramoeba	7+1*	–
	Pseudoparamoeba	17	1
	Vexillifera	13	1
	Clydonella	1+1*	1*
	Paravannella	3+1*	–
	Ripella	14	–
	Vannella	79+2*	2+2*
	Thecamoeba	1*	–
Centramoebia	*Acanthamoeba*	106+1*	–
	Balamuthia	7	–
	Cochliopodium	74	–
	Ovalopodium	3	–
	Parvamoeba	2	–
	Planopodium	3	–
Echinamoebida	*Vermamoeba*	3*	–
Euamoebida	*Copromyxa*	4	–
	Saccamoeba	1	–
Arcellinida	*Alabasta*	4	–
	Alocodera	3	–
	Apodera	6	–
	Awerintzewia	1	–
	Bullinularia	2	–
	Centropyxis	1	–
	Certesella	1	–
	Cornutheca	4	–
	Cylindrifflugia	9	–
	Difflugia	5	–
	Gibbocarina	3	–
	Golemanskia	2	–
	Hyalosphenia	463	298 (*H. papilio*)
	Longinebela	11	–
	Mrabella	3	–
	Nebela	121**	1 (*N. jiuhuensis*)
	Padaungiella	1	–
	Planocarina	23	–
	Quadrulella	40	–
	Trigonopyxis	1	–
	Arcella	13	–
	Cucurbitella	4	–
	Galeripora	26	–
	Netzelia	3	–
	Zivkovicia	7	–
Cutosea	*Squamamoeba*	5	–
Dictyostelia	*Dictyostelium*	78+4*	–
	Polysphondylium	2*	–

Table 1. Continuation.

Myxogastria	*Arcyria*	2	–
	Badhamia	2	2
	Clastoderma	2	–
	Diachea	3	3
	Diderma	6	3
	Didymium	9	3
	Fuligo	1	1
	Lepidoderma	19	–
	Lycogala	2	1
	Perichaena	1	–
	Physarum	210	3
	Reticularia	1	1
	Stemonitis	4	2
	Stemonitopsis	1	1
	Trichia	13	–
Variosea	*Phalansterium*	1*	–
	Planoprotostelium	1*	1*
	Protostelium	1*	1*
Unidentified Amoebozoa		3	1
Total		**1578**	**388**

Notes: *Number of whole mitochondrial genomes. **Including environmental samples.

other myxomycetes (Gott et al., 1993; Horton and Landweber, 2000). Recently, RNA editing in the Cox1 gene was experimentally shown by González-Miguéns et al. (2022) for Difflugiidae (Arcellinida). Although these cases were thoroughly documented, other putative cases of RNA editing in barcoding studies were just suggested on the basis of alignments and in silico translation of newly obtained sequences. For example, it is suggested that insertional editing is involved in the genus *Vannella* (e.g. Nassonova et al., 2010; Bondarenko et al., 2021; Kudryavtsev, 2022), but, to my knowledge, has never been proven experimentally. Interestingly, the perception of this putative editing in NCBI has changed with time. Nassonova et al. (2010) published Cox1 sequences (NCBI/GenBank® accession numbers GQ354136-GQ354206) with the putative insertion of a cytosine residue in only one position. These sequences were accepted and verified, although the editing pattern was not tested experimentally. At the same time Cox1 gene sequence of *Vannella* sp. MV4 MH535947 (English et al., 2019) contains three sites where C- or U-insertions may occur. This looks like a significant reading frame shift, as the sequence was obtained from the genomic DNA without experimental evidence for insertional editing. Consequently, the sequence is marked as UNVERIFIED. A case of putative editing was described in a clade of *Vannella samoroda* and *V. ebro* where the latter species contains 12 putative editing sites in the Cox1 gene (Kudryavtsev et al., 2019). However, this sequence was correctly annotated and verified in NCBI. Among 51 *Korotnevella* sequences with UNVERIFIED status, only 11 have single nucleotide deletions that may cause reading frame shifts. Detailed analysis shows that these deletions are likely caused by polymerase errors, as all of them occur in a fraction of different molecular clones of the same PCR product, and the majority looks like deletions of a timidine in poly-T areas (Zlatogursky et al., 2016). Yet, even though the remaining 40 sequences do not contain any indels or reading frame shifts, they are marked as UNVERIFIED. Sequences of *Korotnevella* spp. by Udalov et al. (2020), on the other hand, are deposited normally, despite showing no differences in features from the above-mentioned 40 UNVERIFIED sequences. Among unverified sequences of *Neoparamoeba*, *Pseudoparamoeba*, and *Vexillifera*, no reading frame shifts were detected, except in *Neoparamoeba* sp. 73BVA, MH535939 (English et al., 2019), so the reason why they got this status is unclear. Interestingly, a large number of *Hyalosphenia papilio* sequences with UNVERIFIED status published by Heger et al. (2013) contained eight single nucleotide deletions that caused the reading frame shift. At

the same time, sequences of the same species published later (Gomaa et al., 2014; Oliverio et al., 2015; Mulot et al., 2017; Singer et al., 2019) and containing the same deletions were deposited without this status. Besides, some of the sequences published by Oliverio et al. (2015) do not contain the above-mentioned deletions. A similar reason likely causes UNVERIFIED status for the Cox1 sequence of *Nebela jiuhuensis* (Qin et al., 2016). A total of 20 Cox1 sequences from species of different genera of Myxomycetes are deposited with UNVERIFIED status likely due to RNA editing that causes frame shifts when sequence of genomic DNA is analyzed. Full mitochondrial genomes get this status due to frame shifts in other genes, as neither frame shifts nor signs of RNA editing occur in the Cox1 gene sequences of these genomes.

The above analysis shows that the major challenge in creation of a public reference Cox1 database for Amoebozoa based on the NCBI/GenBank® records is posed by the putative cases of RNA editing that may occur in this marker. Researchers who obtain and deposit these sequences are mostly not in a position to verify every single case of such editing experimentally; however, the deposition of a sequence with UNVERIFIED status makes it virtually invisible for the database users. This may affect evaluation and analysis of further results. The main problem is that, even though the proportion of sequences deposited as UNVERIFIED is currently low, all species in some of the lineages may show this editing. In this case, entire genera may theoretically be invisible in BLAST databases. The most surprising is that part of the sequences demonstrating the same features (i.e. putative editing that is seen as frame shifts at the genomic level) are deposited normally. This lack of uniformity in the deposition policy is confusing and misleading.

Facing these difficulties, I tried to find a way to overcome this problem by communicating with Genbank staff and discussing the situation. As the result of this discussion, I would like to suggest an approach for the researchers who need to submit Cox1 sequences with unusual features such as putative editing seen in Amoebozoa to the NCBI/GenBank® to create a fully searchable public reference database. The recommendation received from the GenBank staff suggests that if sequences with such features are to be submitted, the note indicating putative insertional editing should be added to the Qualifiers. If the submitter does not have experimental evidence for the editing, references to the corresponding studies on similar groups should be provided during subsequent communication with GenBank staff. In this case sequences are accepted in the normal status (i.e. searchable by BLAST). I followed these recommendations with the recent submission of the Cox1 sequences OP080613-OP080627 of *Vannella robusta* Kudryavtsev, 2022, which are identical to the Cox1 sequence of *Vannella* sp. MV4 deposited as UNVERIFIED (English et al., 2019; Kudryavtsev, 2022). I would like to encourage other researchers obtaining Cox1 gene sequences of Amoebozoa to follow these recommendations so that the sequences do not obtain 'UNVERIFIED' status and become visible in BLAST databases. This will improve the functionality of NCBI/GenBank® as a reference Cox1 database for Amoebozoa.

Acknowledgements

Sequence analysis was performed using a computational cluster of the Zoological Institute of the Russian Academy of Sciences which is part of the Shared Equipment Center "Taxon" (https://www.ckp-rf.ru/ckp/3038/). Supported by the Russian Science Foundation grant 20-14-00181.

References

Adl S.M., Bass D., Lane C.E., Lukeš J. et al. 2019. Revisions to the classification, nomenclature, and diversity of eukaryotes. J. Eukaryot. Microbiol. 66 (1): 4–119. https://doi.org/10.1111/jeu.12691

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3): 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Begerow D., Nilsson H., Unterseher M. and Maier W. 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. App. Microbiol. Biotechnol. 87 (1): 99–108. https://doi.org/10.1007/s00253-010-2585-4

Bondarenko N., Smirnov A., Nassonova E., Glotova A. and Fiore-Donno A.-M. 2019. Mitochondrial genomes of Amoebozoa. Protistology. 13 (4): 179–191. https://doi.org/10.21685/1680-0826-2019-13-4-1

Bondarenko N.I., Glotova A.A., Nassonova E.S., Masharski A. and Smirnov A.V. 2021. The complete mitochondrial genome of an unusual strain of tiny vannellid amoeba (Amoebozoa, Discosea,

Vannellida) isolated from the Niagara River (Canada). Protistology. 15 (4): 304–311. https://doi.org/10.21 685/1680-0826-2021-15-4-8

Bondarenko N.I., Nassonova E.S., Mesentsev Y.S. and Smirnov A.V. 2022. Mitochondrial genome of *Thecamoeba quadrilineata* – the first mt genome among the representatives of the order Thecamoebida (Amoebozoa, Discosea). Protistology. 16 (2): 135–142. https://doi.org/10.21685/1680-0826-2022-16-2-7

Bortnikov F.M., Shchepin O.N., Gmoshinskiy V.I., Prikhodko I.S. and Novozhilov Y.K. 2018. *Diderma velutinum*, a new species of *Diderma* (Myxomycetes) with large columella and triple peridium from Russia. Botanica Pacifica. 7 (2): 47–51. https://doi.org/10.17581/bp.2018.07207

Brown W.M. 1985. The mitochondrial genome of animals. In: Molecular evolutionary genetics (Ed: Maclntyre R.J.). Plenum Press, New York, pp. 95–130.

CBOL Plant Working Group 2009. A DNA barcode for land plants. Proc. Natl. Acad. Sci. USA, 106 (31): 12794–12797. https://doi.org/10.1073/pnas.0905845106

DeSalle R. and Goldstein P. 2019. Review and interpretation of trends in DNA barcoding. Front. Ecol. Evol. 7: 302. https://doi.org/10.3389/fevo.2019.00302

Douglas T.E., Kronforst M.R., Queller D.C. and Strassmann J.E. 2011. Genetic diversity in the social amoeba *Dictyostelium discoideum*: Population differentiation and cryptic species. Mol. Phylogenet. Evol. 60 (3): 455–462. https://doi.org/10.1016/j.ympev.2011.05.007

Duckert C., Blandenier Q., Kupferschmid F.A., Kosakyan A. et al. 2018. En garde! Redefinition of *Nebela militaris* (Arcellinida, Hyalospheniidae) and erection of *Alabasta* gen. nov. Eur. J. Protis-tol. 66: 156–165. https://doi.org/10.1016/j.ejop.2018.08.005

Duckert C., Blandenier Q., McKeown M., Hohaia H. et al. 2021. Superficially described and ignored for 92 years, rediscovered and emended: *Apodera angatakere* (Amoebozoa: Arcellinida: Hyalospheniformes) is a new flagship testate amoeba taxon from Aotearoa (New Zealand). J. Eukaryot. Microbiol. 68 (6): e12867. https://doi.org/10.1111/jeu.12867

English C. J., Tyml T., Botwright N. A., Barnes A. C. et al. 2019. A diversity of amoebae colonise the gills of farmed Atlantic salmon (*Salmo salar*) with amoebic gill disease (AGD). Eur. J. Protistol. 67: 27–45. https://doi.org/10.1016/j.ejop.2018.10.003

Feng Y. and Schnittler M. 2015. Sex or no sex? Group I introns and independent marker genes reveal the existence of three sexual but reproductively isolated biospecies in *Trichia varia* (Myxomycetes). Org. Divers. Evol. 15(4): 631–650. https://doi.org/10.1007/s13127-015-0230-x

Folmer O., Hoeh W.R., Black M.B. and Vrijenhoek R.C. 1994. Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. Mol. Mar. Biol. Biotechnol. 3 (5): 294–299.

Fučíková K. and Lahr D.J. 2016. Uncovering cryptic diversity in two amoebozoan species using complete mitochondrial genome sequences. J. Eukaryot. Microbiol. 63 (1): 112–122. https://doi.org/10.1111/jeu.12253

Geisen S., Kudryavtsev A., Bonkowski M. and Smirnov A. 2014. Discrepancy between species borders at morphological and molecular levels in the genus *Cochliopodium* (Amoebozoa, Himatismenida), with the description of *Cochliopodium plurinucleolum* n. sp. Protist. 165 (3): 364–383. https://doi.org/10.1016/j.protis.2014.04.002

Gomaa F., Kosakyan A., Heger T.J., Corsaro D. et al. 2014. One alga to rule them all: unrelated mixotrophic testate amoebae (Amoebozoa, Rhizaria and Stramenopiles) share the same symbiont (Trebouxiophyceae). Protist. 165 (2): 161–176. https://doi.org/10.1016/j.protis.2014.01.002

González-Miguéns R., Todorov M., Blandenier Q., Duckert C. et al. 2022. Deconstructing *Difflugia*: The tangled evolution of lobose testate amoebae shells (Amoebozoa: Arcellinida) illustrates the importance of convergent evolution in protist phylogeny. Mol. Phylogenet. Evol. 175: 107557. https://doi.org/10.1016/j.ympev.2022.107557

Gott J.M., Visomirski L.M. and Hunter J.L. 1993. Substitutional and insertional RNA editing of the cytochrome c oxidase subunit 1 mRNA of *Physarum polycephalum*. J. Biol. Chem. 268 (34): 25483–25486. https://doi.org/10.1016/S0021-9258(19)74417-2

Gouy M., Tannier E., Comte N. and Parsons D.P. 2021. Seaview version 5: A multiplatform software for multiple sequence alignment, molecular phylogenetic analyses, and tree reconciliation. In: Multiple sequence alignment. Methods in molecular biology (Ed: Katoh K.). Humana, New York, pp. 241–260. https://doi.org/10.1007/978-1-0716-1036-7_15

Hansen H., Botwright N.A., Cook M.T., Douglas A. et al. 2019. Genetic diversity among geographically distant isolates of *Neoparamoeba peru-*

rans. Dis. Aquat. Org. 137 (2): 81–87. https://doi.org/10.3354/dao03433

Hebert P.D., Cywinska A., Ball S.L. and De Waard J.R. 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. London. Series B: Biological Sciences. 270 (1512): 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert P.D.N., Ratnasingham S. and De Waard J.R. 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. London. Series B: Biological Sciences. 270 (Suppl. 1): S96–S99. https://doi.org/10.1098/rsbl.2003.0025

Hebert P.D. and Gregory T.R. 2005. The promise of DNA barcoding for taxonomy. Syst. Biol. 54 (5): 852–859. https://doi.org/10.1080/10635150500354886

Heger T.J., Mitchell E.A. and Leander B.S. 2013. Holarctic phylogeography of the testate amoeba *Hyalosphenia papilio* (Amoebozoa: Arcellinida) reveals extensive genetic diversity explained more by environment than dispersal limitation. Mol. Ecol. 22 (20): 5172–5184. https://doi.org/10.1111/mec.12449

Horton T.L. and Landweber L.F. 2000. Evolution of four types of RNA editing in myxomycetes. Rna. 6 (10): 1339–1346. https://doi.org/10.1017/S135583820000087X

Katoh K. and Standley D.M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4): 772–780. https://doi.org/10.1093/molbev/mst010

Kosakyan A., Heger T.J., Leander B.S., Todorov M. et al. 2012. COI barcoding of Nebelid testate amoebae (Amoebozoa: Arcellinida): extensive cryptic diversity and redefinition of the Hyalospheniidae Schultze. Protist. 163 (3): 415–434. https://doi.org/10.1016/j.protis.2011.10.003

Kosakyan A., Gomaa F., Mitchell E.A., Heger T.J. and Lara E. 2013. Using DNA-barcoding for sorting out protist species complexes: a case study of the *Nebela tincta–collaris–bohemica* group (Amoebozoa; Arcellinida, Hyalospheniidae). Eur. J. Protistol. 49 (2): 222–237. https://doi.org/10.1016/j.ejop.2012.08.006

Kosakyan A., Mulot M., Mitchell E.A. and Lara E. 2015. Environmental DNA COI barcoding for quantitative analysis of protists communities: A test using the *Nebela collaris* complex (Amoebozoa; Arcellinida; Hyalospheniidae). Eur. J. Protistol. 51 (4): 311–320. https://doi.org/10.1016/j.ejop.2015.06.005

Kosakyan A., Lahr D.J., Mulot M., Meisterfeld R. et al. 2016. Phylogenetic reconstruction based on COI reshuffles the taxonomy of hyalosphenid shelled (testate) amoebae and reveals the convoluted evolution of shell plate shapes. Cladistics. 32 (6): 606–623. https://doi.org/10.1111/cla.12167

Kostka M., Lares-Jiménez L. F., Tyml T. and Dyková I. 2017. *Copromyxa laresi* n. sp. (Amoebozoa: Tubulinea) and transfer of *Cashia limacoides* (Page, 1967) to *Copromyxa* Zopf, 1885. J. Eukaryot. Microbiol. 64 (2): 173–182. https://doi.org/10.1111/jeu.12349

Kudryavtsev A. 2014. *Paravannella minima* n. g. n. sp.(Discosea, Vannellidae) and distinction of the genera in the vannellid amoebae. Eur. J. Protistol. 50 (3): 258–269. https://doi.org/10.1016/j.ejop.2013.12.004

Kudryavtsev A. 2022. From Argentine Abyssal Plain to farmed turbot in Spain: A ubiquitous amoeba species *Vannella robusta* sp. nov. (Amoebozoa, Vannellida). Eur. J. Protistol. 85: 125912. https://doi.org/10.1016/j.ejop.2022.125912

Kudryavtsev A. and Gladkikh A. 2017. Two new species of *Ripella* (Amoebozoa, Vannellida) and unusual intragenomic variability in the SSU rRNA gene of this genus. Eur. J. Protistol. 61 (A): 92–106. https://doi.org/10.1016/j.ejop.2017.09.003

Kudryavtsev A. and Pawlowski J. 2013. *Squamamoeba japonica* n. g. n. sp. (Amoebozoa): a deep-sea amoeba from the Sea of Japan with a novel cell coat structure. Protist. 164 (1): 13–23. https://doi.org/10.1016/j.protis.2012.07.003

Kudryavtsev A. and Pawlowski J. 2015. *Cunea* n. g. (Amoebozoa, Dactylopodida) with two cryptic species isolated from different areas of the ocean. Eur. J. Protistol. 51 (3): 197–209. https://doi.org/10.1016/j.ejop.2015.04.002

Kudryavtsev A., Pawlowski J. and Smirnov A. 2018. More amoebae from the deep-sea: Two new marine species of *Vexillifera* (Amoebozoa, Dactylopodida) with notes on taxonomy of the genus. Eur. J. Protistol. 66: 9–25. https://doi.org/10.1016/j.ejop.2018.07.001

Kudryavtsev A., Völcker E., Clauß S. and Pawlowski J. 2021. *Ovalopodium rosalinum* sp. nov., *Planopodium haveli* gen. nov, sp. nov., *Planopodium desertum* comb. nov. and new insights into phylogeny of the deeply branching members of the order Himatismenida (Amoebozoa). Int. J. Sys. Evol. Microbiol. 71 (3): 004737. https://doi.org/10.1099/ijsem.0.004737

Kudryavtsev A. and Volkova E. 2018. *Clydonella sawyeri* n. sp. (Amoebozoa, Vannellida): mor-

phological and molecular study and a re-definition of the genus *Clydonella* Sawyer, 1975. Eur. J. Protistol. 63: 62–71. https://doi.org/10.1016/j.ejop.2018.01.008

Kudryavtsev A., Volkova E. and Plotnikov A. 2019. *Vannella samoroda* n. sp. (Amoebozoa) – first member of the genus from a continental saline habitat placed in a molecular tree. Eur. J. Protistol. 71: 125634. https://doi.org/10.1016/j.ejop.2019.125634

Kudryavtsev A. and Volkova E. 2020. *Cunea russae* n. sp. (Amoebozoa, Dactylopodida), another cryptic species of *Cunea* Kudryavtsev and Pawlowski, 2015, inhabits a continental brackish-water biotope. Eur. J. Protistol. 73: 125685. https://doi.org/10.1016/j.ejop.2020.125685

Kudryavtsev A.A., Volkova E.N. and Voytinsky F.P. 2020. Morphological and molecular investigation of *Vexillifera* cf. *armata* Page, 1979 (Amoebozoa: Dactylopodida) isolated from the Pacific Ocean. Invert. Zool. 17 (4): 385–402. https://doi.org/10.15298/invertzool.17.4.04

Liu Q.S., Yan S.Z. and Chen S.L. 2015. Species diversity of myxomycetes associated with different terrestrial ecosystems, substrata (microhabitats) and environmental factors. Mycol. Prog. 14 (5): 1–13. https://doi.org/10.1007/s11557-015-1048-9

Mulot M., Marcisz K., Grandgirard L., Lara E. et al. 2017. Genetic determinism vs. phenotypic plasticity in protist morphology. J. Eukaryot. Microbiol. 64 (6): 729–739. https://doi.org/10.1111/jeu.12406

Nassonova E., Smirnov A., Fahrni J. and Pawlowski J. 2010. Barcoding amoebae: comparison of SSU, ITS and COI genes as tools for molecular identification of naked lobose amoebae. Protist. 161 (1): 102–115. https://doi.org/10.1016/j.protis.2009.07.003

Oliverio A.M., Lahr D.J., Grant J. and Katz L.A. 2015. Are microbes fundamentally different than macroorganisms? Convergence and a possible case for neutral phenotypic evolution in testate amoeba (Amoebozoa: Arcellinida). R. Soc. Open Sci. 2 (12): 150414. https://doi.org/10.1098/rsos.150414

Pawlowski J., Audic S., Adl S., Bass D., et al. 2012. CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. PLoS biology. 10 (11): e1001419. https://doi.org/10.1371/journal.pbio.1001419

Pombert J.F., Smirnov A., James E.R., Janouškovec J. et al. 2013. The complete mitochondrial genome from an unidentified *Phalansterium* species. Protist Genomics. 1: 25–32. https://doi.org/10.2478/prge-2013-0002

Qin Y., Man B., Kosakyan A., Lara E. et al. 2016. *Nebela jiuhuensis* nov. sp. (Amoebozoa; Arcellinida; Hyalospheniidae): a new member of the *Nebela saccifera-equicalceus-ansata* group described from *Sphagnum* peatlands in south-central China. J. Eukaryot. Microbiol. 63 (5): 558–566. https://doi.org/10.1111/jeu.12300

Sayers E.W., Bolton E.E., Brister J.R., Canese K. et al. 2022. Database resources of the national center for biotechnology information. Nucleic Acids Res. 50 (D1): D20–D26. https://doi.org/10.1093/nar/gkab1112

Schoch C.L., Seifert K.A., Huhndorf S., Robert V. et al. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. USA 109 (16): 6241–6246. https://doi.org/10.1073/pnas.1117018109

Seifert K.A. 2009. Progress towards DNA barcoding of fungi. Mol. Ecol. Res. 9 (s1): 83–89. https://doi.org/10.1111/j.1755-0998.2009.02635.x

Shchepin O.N., Dagamac N.H., Sanchez O.M., Novozhilov Y.K. et al. 2017. DNA barcoding as a tool for identification of plasmodia and sclerotia of myxomycetes (Myxogastria) appearing in moist chamber cultures. Mycosphere. 8 (10): 1904–1913. https://doi.org/10.5943/MYCOSPHERE/8/10/13

Shchepin O., Novozhilov Y., Woyzichovski J., Bog M. et al. 2022. Genetic structure of the protist *Physarum albescens* (Amoebozoa) revealed by multiple markers and genotyping by sequencing. Mol. Ecol. 31 (1): 372–390. https://doi.org/10.1111/mec.16239

Singer D., Kosakyan A., Pillonel A., Mitchell E.A. and Lara E. 2015. Eight species in the *Nebela collaris* complex: *Nebela gimlii* (Arcellinida, Hyalospheniidae), a new species described from a Swiss raised bog. Eur. J. Protistol. 51 (1): 79–85. https://doi.org/10.1016/j.ejop.2014.11.004

Singer D., Mitchell E.A.D., Payne R.J., Blandenier Q. et al. 2019. Dispersal limitations and historical factors determine the biogeography of specialized terrestrial protists. Mol. Ecol. 28 (12): 3089–3100. https://doi.org/10.1111/mec.15117

Tekle Y.I. 2014. DNA barcoding in Amoebozoa and challenges: the example of *Cochliopodium*. Protist. 165 (4): 473–484. https://doi.org/10.1016/j.protis.2014.05.002

Traphagen S.J., Dimarco M.J. and Silliker M.E. 2010. RNA editing of 10 *Didymium iridis* mito-

chondrial genes and comparison with the homologous genes in *Physarum polycephalum*. RNA. 16: 828–838. https://doi.org/10.1261/rna.1989310

Udalov I.A., Lotonin K. and Volkova E. 2020. Description of a new species of marine amoeba *Korotnevella mutabilis* n. sp. (Amoebozoa, Dactylopodida). Eur. J. Protistol. 75: 125701. https://doi.org/10.1016/j.ejop.2020.125701

Udalov I.A. and Völcker E. 2022. Is scale's structure still a good character to delimitate species of Amoebozoa? case of the genus *Korotnevella* (Amoebozoa, Dactylopodida). Org. Divers. Evol. 22 (1): 17–33. https://doi.org/10.1007/s13127-021-00521-z

Volkova E., Völcker E., Claulß S., Bondarenko N. and Kudryavtsev A. 2019. *Paramoeba aparasomata* n. sp., a symbiont-free species, and its relative *Paramoeba karteshi* n. sp. (Amoebozoa, Dactylopodida). Eur. J. Protistol. 71: 125630. https://doi.org/10.1016/j.ejop.2019.125630

Zlatogursky V.V., Kudryavtsev A., Udalov I.A., Bondarenko N. et al. 2016. Genetic structure of a morphological species within the amoeba genus *Korotnevella* (Amoebozoa: Discosea), revealed by the analysis of two genes. Eur. J. Protistol. 56: 102-111. https://doi.org/10.1016/j.ejop.2016.08.001